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From: Gabel, Gailene
Sent: Tuesday, March 02, 2004 7:09 PM
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Subject: 09/511,824

Please provide a copy of the following literature ASAP:

1) Winkles J et al., Enhanced-latex-agglutination assay for C-reactive protein in serum, with use of a centrifugal analyzer. Clinical chemistry, (1987 May) 33 (5) 685-9.

2) Kitahashi S. et al.; Diagnosis of infections in newborns using a new particle-mediated immunoassay for serum C-reactive protein. Journal of Automatic Chemistry, (1998) 20/6 (195-198).

3) Ueno T. et al., Liposome turbidimetric assay (LTA). Advanced Drug Delivery Reviews, (1997) 24/2-3 (293-299).

thanks a bunch,
Gailene R. Gabel
Patent Examiner
Art Unit 1641
(571) 272-0820
Remsen E03D64

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Enhanced-Latex-Agglutination Assay for C-Reactive Protein in Serum, with Use of a Centrifugal Analyzer

J. Winkles,[1] J. Lunec,[1,3] and I. Deverill[2]

This is an improved assay of C-reactive protein in serum, for use with the Baker "Encore" centrifugal analyzer. Features of this assay include: 250-specimen throughput per hour, within-batch CV 2.2%, between-batch CV 2.7%, no antigen-excess problems up to 1000 mg/L, negligible interference from rheumatoid factor, and good correlation ($r = 0.99$) with radial immunodiffusion. The method is inexpensive and automated, involving no predilution steps. It can be adapted for use in a wide range of systems and can be used for single urgent estimations.

Additional Keyphrases: *acute-phase proteins · radial immunodiffusion compared*

C-reactive protein (CRP; M_r 110 000–140 000), the classic acute-phase protein of human serum, is synthesized by hepatocytes. Normally, it is present only in trace amounts in serum, but it can increase by as much as 1000-fold in response to injury or infection (*1*, *2*). In vitro, complexes with CRP can activate the complement system, promote phagocytosis, inhibit platelet aggregation, and interact with and activate certain sub-populations of lymphocytes (*3–5*). Despite these findings, the actual biological function of CRP has not yet been defined, although it probably acts primarily as a protective agent during the onset of inflammation and tissue damage. The clinical measurement of CRP in serum therefore appears to be a valuable screening test for organic disease and a sensitive index of disease activity in inflammatory, infective, and ischemic conditions. In rheumatology, CRP seems to be the best single parameter for estimating disease activity and response to therapy (*6*). CRP is now increasingly quantified to differentiate bacterial from viral infections (*7*, *8*), and it can also be useful in diseases such as myocardial infarction, appendicitis, and postoperative complications (*1*). Given this wide role of CRP in clinical diagnosis and treatment, a reliable, inexpensive automated method is needed for its determination in biological fluids.

Here we report a method in which antibodies to human CRP are simply adsorbed to latex particles and uncoated latex sites are saturated with bovine serum albumin. The tendency of the coated latex particles to aggregate in the absence of CRP antigen, particularly during the process of preparing the stock reagent, is alleviated by sonication. The assay consists of monitoring the increase in turbidity that results from the interaction of coated latex beads with CRP at optimum conditions.

[1] Division of Rheumatology Biochemistry, Department of Biochemistry, Selly Oak Hospital, Raddlebarn Road, Birmingham B29 6JD, U.K.

[2] Department of Immunology, University Hospital, Queens Medical Centre, Nottingham NG7 2UH, U.K.

[3] Address correspondence to this author.

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Materials and Methods

Apparatus. We used an "Encore" centrifugal analyzer with its associated P1000 sampler (Baker Instruments Ltd., Egham, U.K.), a Heraeus Christ minifuge GL (VA Howe Ltd., London, U.K.), and a probe sonicator (Model A180G; Ultrasonics Ltd., Shipley, U.K.) in this study.

Reagents and standards. Latex "polybead" polystyrene microspheres (25 g/L suspension), 57 (SD 5) nm in diameter, were obtained from Polysciences Ltd., Northhampton, U.K. Antiserum to human CRP, raised in sheep, was from Unipath Ltd., Bedford, U.K. Bovine serum albumin, 220 g/L, in isotonic saline (sodium chloride 8.5 g/L), was from Sigma Ltd., Dorset, U.K. CRP standard material and radial immunodiffusion plates were from Hoechst Ltd., Middlesex, U.K. All other reagents were of "Analar" grade and were from BDH Ltd., Atherstone, U.K.

A specimen of pooled serum with high CRP concentration (112 mg/L) was calibrated against the commercial preparation and diluted in phosphate-buffered isotonic saline, pH 7.4. This was used to prepare the standard curve.

Samples. Blood was sampled from patients in rheumatology clinics at Selly Oak Hospital and from presumably healthy blood donors.

Preparation and stock latex reagent. We added 250 μL of anti-CRP antibody to 10 mL of chilled (4 °C) glycine-buffered saline (pH 8.2, containing, per liter, 97.5 mmol of NaCl, 2.5 mmol of NaOH, and 97.5 mmol of glycine), then added 1 mL of latex beads. After promptly mixing the contents of the tube by shaking, we surrounded the tube with ice, and placed it on a mechanical rotor for 60 min. We then centrifuged the contents of the tube (4000 × *g*, 4 °C) for 10 min or until a clear supernate was obtained, which we discarded. We added to the latex pellet 10 mL of chilled glycine-buffered saline, this time at pH 10.0 and containing 63 mmol of NaCl, 37 mmol of NaOH, and 63 mmol of glycine per liter, then added 100 μL of the bovine serum albumin preparation to block uncoated latex binding sites and stabilize the reagent. The latex pellet was resuspended by vortex-mixing, then sonicated (by using a 1-cm probe with power and tuning dials set at six) until a clear, pale-amber colloidal solution was obtained (typically 30–40 s). We were careful to see that the solution did not become overheated. Sonicating the stock latex for longer than about 60 s can cause the preparation to overheat, with consequent denaturation of both the CRP antibody and the albumin that is used to block the uncoated binding sites. However, this effect is easily detected because the latex flocculates and comes out of solution. Such reagent must be discarded; further sonication, even after cooling, will not restore the desired solution. If the tube, latex pellet, and glycine-buffered saline are all chilled to 4 °C before sonication, this problem will not be encountered before at least 60 s, i.e., about twice the recommended sonication period. Undersonication of the stock latex will not produce the clear-amber colloidal solution, and there will be a subsequent loss in assay sensitivity; further sonication, however, will produce the required conditions. This amount of stock latex preparation suffices for about 200 analyses.

Measurement of CRP. A working latex reagent was prepared by diluting the stock reagent sixfold with pH 10 glycine-buffered saline. We added 2 μL of sample, standard, or control to 300 μL of working reagent, using 18 μL of distilled water as a diluent to prevent carryover by the sample probe. In the Encore analyzer, the solutions were mixed for 1.6 s. After 4 s, a blank reading was taken at 340 nm. The absorbance was monitored at 340 nm at 2-s intervals between 100 and 170 s, and the mean absorbance was calculated. Results were calculated from this mean absorbance minus the 4-s blank absorbance. Table 1 lists the instrument settings for the P1000 sampler and the analyzer. For CRP controls we diluted the commercial serum preparations with phosphate-buffered saline to give five different concentrations. Serum samples were obtained without preservative, stored at 4 °C, and either analyzed within seven days or stored at −20 °C until assay. In a separate experiment, we found CRP to be stable for at least three months at −20 °C.

Interference from rheumatoid factor. A serum with known high content of rheumatoid factor (Rose–Waaler titer, 1/1024) was diluted 16-fold with chilled distilled water and left at 4 °C for 60 min. After centrifugation and discarding the supernate, we redissolved the pellet to its original serum volume in phosphate-buffered saline. We checked the purity of a sample of this solution by use of liquid chromatography. Different amounts of this preparation were added to the reaction mixture (both before and after treatment with DL-dithiothreitol to destroy rheumatoid factor by reducing its disulfide bonds) and the reaction was monitored. Commercial CRP was also diluted with this preparation and assayed. The regression analysis between results by our latex method and those by radial immunodiffusion involved both latex-positive samples and samples that were negative for rheumatoid factor.

Analytical Variables

Reproducibility and stability of latex reagent. We assayed a diluted preparation of CRP (Hoechst) in phosphate-buffered saline in 12 different rotors, on 16 separate days during two months. This involved the use of at least 10 different batches of stock latex, each batch used either on the day of preparation or stored at 4 °C and used within one week. The mean CRP concentration measured was 51.7 (SD 1.4) mg/L (CV 2.8%). The same preparation of CRP was used during the −20 °C stability experiment for the stock latex (see

Table 1. Instrument Settings for the Baker Encore and P1000

Baker Encore	
Test type	D (specific protein)
Reaction direction	A (increasing)
Low-pass	A (low)
Analytical wavelength	B (340 nm)
Blank wavelength	B (340 nm)
Blank mode	C (timed)
Blank time	4 (seconds)
Initial time	100 (seconds)
Time window	70 (seconds)
Final time	180 (seconds)
Mix time	1.6 (seconds)
Linearity	5.0 (milli-absorbance units)
Abnormal absorbance limit	1.0 (absorbance units)
Light level	A (normal)
Optical signal	A (absorbance)
y-Transform	A (none)
Curve fit	C3 (polynomial order 3)
x-Transform	A (none)
Temperature	C (37 °C)
P1000 Sampler	
Sample vol	2 μL
Diluent vol	18 μL
Reagent vol	300 μL

Figure 1C). The mean concentration measured was 52.3 (SD 1.5) mg/L (CV = 2.8%, n = 9). We conclude that the latex reagent is stable for at least seven days at 4 °C or for 16 weeks at −20 °C. Repeated freeze–thawing of the stock latex reagent results in aggregation of the latex and loss of assay sensitivity.

Optimization, precision, and sensitivity. Optimization of reaction conditions, results of pH changes, antibody loading of latex particles, and stability curves are all illustrated in Figure 1.

Within-batch and between-batch precision were calculated from assays of suitable dilutions of commercial CRP serum in pH 7.4 phosphate-buffered saline (Table 2), performed during one working day with five different batches of stock reagent. The limit of detection (3 SD for the smallest concentration that was distinguishable from the blank) was 3 mg/L, as determined by double-diluting the standard material and assaying each dilution in one batch 22 times.

Accuracy and linearity. The equation for the correlation between results from radial immunodiffusion plates (y) and our method (x) was $y = 0.96x - 1.05$ mg/L (SE = 2.5 mg/L; n = 64; $r = 0.99$). The standard curve is linear to about 80 mg/L and the useful assay range extends to about 112 mg/L. The data are best fitted by a third-order polynomial function (Figure 2, bottom).

Speed of analysis. Twenty-eight samples can be processed on any one transfer disk (including standards and controls). Results are ready within 8 min, including pipetting time.

Detection of antigen excess. All fluid-phase immunochemical assays suffer from the potential for nondetection of antigen excess. Under our reaction conditions, antigen excess is manifested by a gradual decrease in absorbance after the equivalence point has been reached. At CRP concentrations ≤1000 mg/L this stage is not reached until after the assay interval (Figure 3). Thus samples with high concentrations of CRP (≤1000 mg/L) give absorbances exceeding that of the top standard.

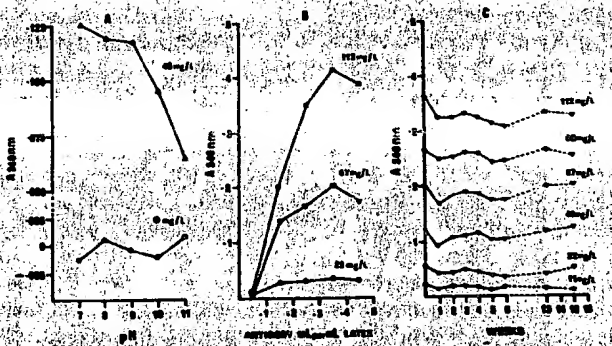

Fig. 1. Optimum reaction and stability conditions for the latex reagent: (*A*) pH of buffer, (*B*) antibody coating (mL of antibody per mL of latex beads), (*C*) reagent stability at −20 °C

Table 2. Precision Data for the CRP Assays

	Samples				
	A	B	C	D	E
Within run (n = 22)					
Mean, mg/L	88.3	43.2	19.1	9.5	4.9
SD, mg/L	0.6	0.9	0.6	0.5	0.6
CV, %	0.9	2.2	3.1	5.5	12.1
Between run (n = 20)					
Mean, mg/L	85.7	43.6	20.8	10.1	5.1
SD, mg/L	1.8	1.2	0.8	0.5	0.6
CV, %	2.1	2.7	3.7	5.2	11.8

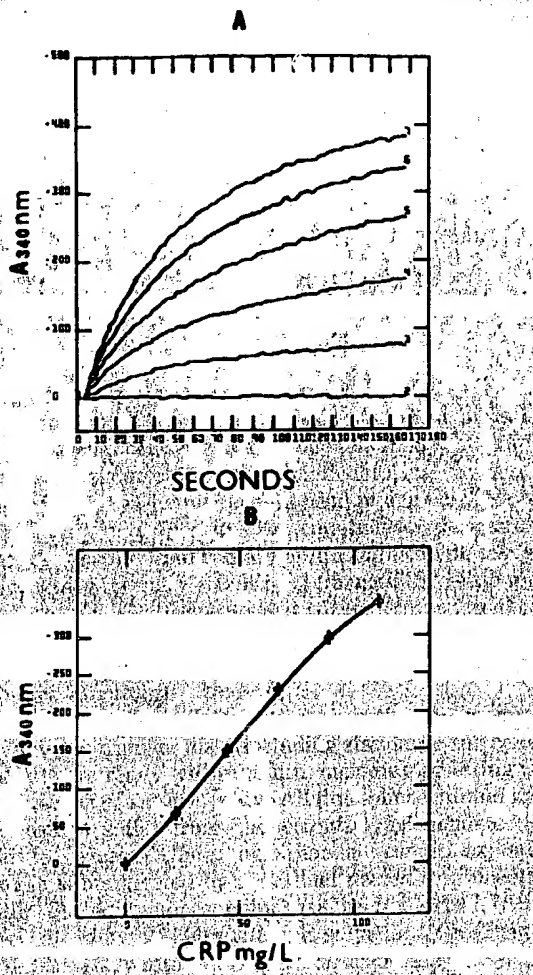

Fig. 2. (*Top*) Kinetics of the reactions of CRP standards during the assay and (*bottom*) the resulting standard curve

Numerals after curves refer to cuvette positions (Baker Encore graphics mode) and can be ignored

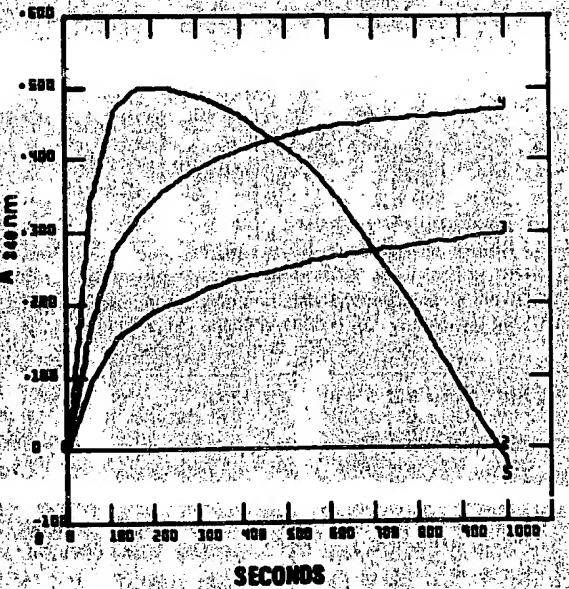

Fig. 3. Reaction kinetics during 1000 s, showing the elimination of antigen-excess problems by our latex method

CRP concentrations assayed were (2) 0, (3) 67, (4) 112, and (5) 1120 mg/L. Numerals after curves refer to cuvette positions

Interference studies. Interference by rheumatoid factor in this method is pH dependent (Figure 4), decreasing with increasing alkalinity up to pH 10, then appearing to increase. This latter effect is possibly ascribable to the decreased sensitivity of the CRP reaction at this pH (Figure 1). A euglobin preparation (Rose–Waaler titer of 1/1024) gave an assay response equivalent to an apparent CRP concentration of about 1 mg/L; this increased proportionately with the Rose–Waaler titer (Figure 4). Analytical recoveries of commercial CRP diluted in the same euglobin precipitate are about 100%.

The correlation coefficient between radial immunodiffusion and our method was calculated for samples that were both latex-positive (n = 28, Rose–Waaler titer 0 to 1/1024) and latex-negative for rheumatoid factor (n = 36). The result (r = 0.99) indicates no interference. Latex-positive (n = 16) and latex-negative samples (n = 86) that were found to be below the limit of detection when measured by radial immunodiffusion (6 mg/L according to Hoechst Ltd.) were assayed by our CRP assay. Latex-positive specimens yielded $\bar{x}$ = 5.4 (SD 1.9) mg/L, latex-negative specimens yielded $\bar{x}$ = 4.7 (SD 1.4) mg/L. This experiment confirmed the lack of interference from antibody to rheumatoid factor.

Hemoglobin (5 g/L), bilirubin (330 μmol/L), or triglyceride (6.78 mmol/L) had no effect on results by our method.

Normal Reference Interval

Of 50 samples from blood donors, assayed by this method, >90% had concentrations below the limit of detection (i.e., <3 mg/L). Therefore, we consider values <3 mg/L to be normal.

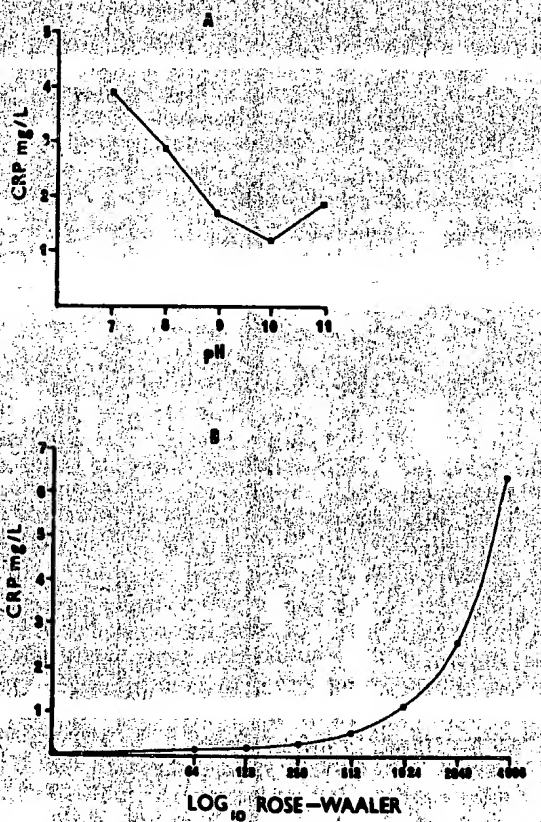

Fig. 4. (*A*) Effect of pH on interference by rheumatoid factor; (*B*) Increase in apparent CRP concentration with increasing Rose–Waaler titer

Discussion

In previous attempts to measure CRP, polyethylene glycol was used to enhance the interaction between antibody and antigen (*9, 10*). However, in that method the antiserum concentration is greater, typically 50-fold diluted antiserum is used, as compared with an approximately 250-fold dilution for our method. When non-enhanced methods are used, relatively large amounts of serum must be included in the reaction mixture. The resulting decrease in precision is attributable to the background turbidity from proteins, triglycerides, and chylomicrons, and there is potential for inaccuracy from interference by rheumatoid factor.

The agglutination of latex particles with antigen or antibody to form large, visible aggregates was originally exploited as a semiquantitative slide test. Latex particles act as inert spacers in the antigen–antibody lattice, thereby increasing the physical size of the immune complexes formed. Latex particle size is important in the assay. Optical methods for measuring aggregation—by changes in absorbance (turbidimetry) or light scattering (nephelometry)—rely on the growth in the size of the complex. However, beyond some finite limit of complex size there is no further increase in light absorbance or scattering, regardless of complex growth. This "light-scattering plateau" depends on factors such as the wavelength of the incident light, particle geometry, and the refractive index of the suspending medium (*11*). In order to have the broadest assay range possible, we used the smallest particle available (57 nm in diameter). At constant weight for volume, suspensions with smaller particle sizes will have greater total surface areas available for coating.

We have in fact chosen a less-than-optimum amount of antibody coating for the reaction, partly for economy (antibody being the single most expensive component of the reagent), and partly because at higher coating concentrations the stock latex suspension became less stable on sonication. We also chose to use a pH that exceeds the optimum for the pH-dependent reaction. Complexes form more rapidly at near-physiological pH, but at this pH rheumatoid factor also interferes more. Use of pH 10 minimizes this interference while still providing good sensitivity for the CRP reaction. The pH appeared to have no effect on the stability of the blank reaction during the assay.

In our own particular application for studies in rheumatology, the nature of the antibody used for the assay appears to be crucial. Most CRP assays include rabbit antisera (IgG). However, the interaction of rheumatoid factor antibody with the Fc portion of rabbit IgG may lead to considerable error in the estimation of CRP in patients with rheumatoid arthritis and associated disorders. In fact, the Rose–Waaler rheumatoid factor assay wholly depends on that reaction. Thus, for our purposes it was essential to use sheep IgG antisera, which react to a lesser extent with antibody to rheumatoid factor (Figure 4).

One reason why latex-enhanced immunochemical tests may be less popular than similar assays is that one must sonicate the reagent, at least at the stock-preparation stage, which can involve a large capital outlay for a sonicator. The tendency of antibody-coated particles to aggregate in the absence of antigen is an especial problem at the coating stage. Under our preparation conditions, the stock latex reagent is stable without further sonication for as long as a week at 4 °C or for at least three months at −20 °C (Figure 1); thus access to a sonicator is only infrequently required.

In summary, this latex-enhanced immunoassay for CRP, developed for use with a centrifugal analyzer, is precise, specific, inexpensive, and automated. It can deal with large workloads (specimen throughput 250 per hour) or single "urgent" assays (result available within 8 min) and is ideal for pediatric work (2-μL samples). It may also be adapted with little modification for manual use or use with other analyzers.

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CLIN. CHEM. 33/5, 689–691 (1987)

Mathematical Treatment of Data for Calculating Activities of α-Amylase Isoenzymes

Francisco L. Redondo

Methods for determining α-amylase isoenzymes by selective inhibition with a wheat-germ protein are practical and easy, and give accurate, precise results. The incomplete specificity of the inhibitory action is not a major drawback but does necessitate mathematical treatment of the data (i.e., enzymic activities measured before and after preincubation with the inhibitor) to ascertain the amount of the different isoamylases. Such an algorithm is quite simple and straightforward, because the isoenzymes can be calculated either arithmetically or geometrically, by using a linear standard curve, empirically obtained, that relates the fraction of activity remaining after inhibition (R/T) to the pancreatic isoenzyme fraction or to the percentage of total α-amylase (P/T or 100 × P/T). An alternative method, plotting R/T against the ratio of pancreatic to salivary isoenzyme (P/S), is inconvenient, necessarily yields a nonlinear curve, needlessly complicates the calculations, and has been a persistent source of confusion in many articles dealing with the differentiation of isoamylases.

Several isoenzymes of α-amylase (EC 3.2.1.1; 1,4-α-D-glucan glucanohydrolase) have been found in various biological tissues and fluids, including serum. Some of the methods for separating the isoenzymes (electrophoresis, isoelectric focusing, chromatography, etc.) are unsuitable for most clinical situations, which demand a fast and practical approach to the differentiation of these isoenzymes. In such situations, measurement of the salivary- and pancreatic-type isoamylases in serum or urine (or both) is usually adequate, the pancreatic type, obviously, being the one to be considered for the diagnosis of pancreatic disease.

Department of Clinical Biochemistry, Hospital Central de Cruz Roja, Av. Reina Victoria, 22-26, 28003 Madrid, Spain.

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Data Processing in Selective Inhibition Methods

To separate these two isoamylases, the method originally described by O'Donnell et al. (*1*) exploits the selective inhibition of the salivary-type amylase by a protein isolated from wheat (*Triticum aestivum*) (*2*). Such inhibition is not completely selective, however, and not all the salivary-type activity disappears, nor does the pancreatic-type activity remain unaffected, after the action of the inhibitor. This is also what happens, by a completely different mechanism, in the more recently described immunological methods, in which a monoclonal antibody is directed against the salivary-type isoamylase (*3*, *4*).

Therefore, because of the incomplete selectivity of inhibition, the remaining activity (R) in any biological sample containing a mixture of both isoamylases has to be estimated from the following equation:

$$R = a \cdot P + b \cdot S \qquad (1)$$

where P and S represent the activity, before inhibition, of the pancreatic and salivary isoenzymes, respectively; a is the fraction of P activity remaining after inhibition; and b is the fraction of S activity remaining after inhibition. The figures for the constants a and b are experimentally obtained for the conditions of the assay by using pure pancreatic and salivary isoenzymes.

For the rest of my exposition, and for the practical validity of the method, some assumptions have to be made and their correctness must be proved in each analytical design: (*a*) the degree of inhibition is constant over the expected range of P and S; (*b*) the inhibition of P is not affected by the presence of S, and vice versa. In other words, a and b are constant under the experimental conditions chosen. A third assumption, already mentioned by O'Donnell et al., is equally important but of a different character: supposedly, total serum amylase consists of pancreatic- and salivary-type isoenzymes only.